Biochem. J. (1974) **140**, 539–548
Printed in Great Britain

Compartmentation of Free Amino Acids for Protein Synthesis in Rat Liver

By JUDITH AIRHART, ALDA VIDRICH and EDWARD A. KHAIRALLAH
Department of Biochemistry and Biophysics, University of Connecticut, Storrs, Conn. 06268, *U.S.A.*

(*Received* 8 *January* 1974)

The concept that a general intracellular pool serves as the sole precursor of amino acids for protein biosynthesis has been vigorously debated in recent years. To help resolve this controversy, we followed the distribution of intraperitoneally administered [^{3}H]valine in the tRNA and the extracellular and intracellular compartments of rat liver. The specific radioactivity of the valine released from isolated tRNA was 2–3 times higher than that of intracellular valine, suggesting that the intracellular pool cannot be the sole precursor of amino acids used for charging tRNA. In addition, the specific radioactivity of the tRNA was only half that of the extracellular valine. Therefore it is unlikely that the valyl-tRNA is charged exclusively with amino acids derived from the extracellular pool. A model is proposed which stipulates that both extracellular and intracellular amino acids contribute to a restricted compartment that funnels amino acids towards protein biosynthesis.

Although the initial steps in protein biosynthesis are known to proceed with great chemical specificity, little information is available about their functional organization within the cell. Activated amino acids cannot be presented to the ribosomal machinery until they have been attached to the appropriate tRNA species by specific aminoacyl-tRNA synthetases. Traditionally amino acid activation has been presumed to occur throughout the cytosol in open competition with other amino acid metabolizing pathways. Since the tRNA species and the components that charge them are isolated as a routine from the 'soluble' fraction of the homogenate, it has been assumed that a common intracellular pool serves as the obligatory source of amino acids for protein biosynthesis.

This concept has been challenged in recent years. Evidence for amino acid compartmentation in a variety of isolated tissues has been based primarily on observations of linear uptake of radioactive amino acids into protein at a time well before the label equilibrated with intracellular amino acids (Kipnis *et al.*, 1961; Manchester & Wool, 1963; Rosenberg *et al.*, 1963; Hider *et al.*, 1969, 1971; vanVenrooij *et al.*, 1972, 1973). This evidence suggests that amino acids from an extracellular precursor pool may funnel directly into the synthetic machinery. However, other investigators have been unable to calculate reasonable rates of protein synthesis when they assumed an extracellular precursor pool and have concluded that the intracellular pool, or some portion of it, best represents the true source of amino acids (Loftfield & Harris, 1956; Mortimore *et al.*, 1972; Henshaw *et al.*, 1971; Morgan & Peters, 1971; Morgan *et al.*, 1971; Alpers & Thier, 1972; Fern & Garlick, 1973; Li *et al.*, 1973). However, because of technical difficulties associated with tissue preparations *in vitro*, some of these results have been subjected to alternative interpretations (Li *et al.*, 1973; Jefferson *et al.*, 1972; Manchester, 1970).

There are several very practical reasons for resolving this controversy. In the first place, to make conclusions about the rate of protein biosynthesis from isotopic tracer incorporation it is essential to monitor label flow through a clearly defined precursor pool. Further, a great deal of evidence has accumulated recently to suggest that the extent of charging of tRNA may co-ordinate a number of critical metabolic activities in mammalian (Smulson & Thomas, 1969; Munro, 1970; Hershko *et al.*, 1971) and bacterial cells (Neidhardt, 1966; Goldberg, 1971; Haseltine & Block, 1973). Therefore as we investigate the site of amino acid acylation we may also be describing an important locus for the nutritional and hormonal regulation of basic cell functions.

If indeed there is effective compartmentation of the pool of free amino acids, then the most direct approach would be to take advantage of the fact that for protein synthesis the amino acids must first become linked to tRNA. Measurements of the specific radioactivities of the amino acids attached to the tRNA *in vivo* should thus provide an approach to indicate the specific radioactivity of the pool from which these amino acids are drawn. Several laboratories have attempted such an approach by isolating the aminoacyl-tRNA from cardiac muscle (Davey & Manchester, 1969; Martin *et al.*, 1973). In the

present paper we give definitive evidence that in mammalian liver the amino acids are funnelled into protein biosynthesis from a restricted amino acid source different from that of the general pool.

Experimental

Male Charles River rats were housed under controlled lighting conditions (dark period 5:30p.m. to 5:30a.m.). Purina rat pellets were given once daily at 6p.m. and the uneaten food was removed at 10p.m. By training rats to consume their daily food intake in one 4h period, we were able to decrease the variation between animals for all parameters studied. Rats were 5–6 weeks old at the time of death and had been maintained on the feeding schedule for at least 7 days. All animals were killed between 9 and 11a.m.

The rats were anaesthetized with Nembutal (5mg/0.5ml per 100g body wt.) and 15min later [^{3}H]valine (33μCi/0.2ml per 100g body wt.) was administered intraperitoneally. [^{3}H]Valine has several advantages as a tracer for liver protein synthesis. Branched-chain amino acids are not synthesized by the rat nor significantly catabolized by the liver (Miller, 1962; Elwyn *et al.*, 1968; Mortimore & Mondon, 1970). Also, because of the high specific radioactivity of tritiated material, the injection of valine (1.1nmol) did not significantly alter the steady-state concentration of valine in the portal plasma (176nmol/ml). The selection of the intraperitoneal route of injection was prompted by our observation that, in contrast with intravenous injections, this route delivered maximal label to the liver and yet the delivery was sufficiently slow to permit accurate observations of the initial distribution of the label.

At selected times after the administration of the label, the abdominal cavity was opened and blood was withdrawn from the portal vein proximal to the liver; the thoracic cavity was opened and blood was withdrawn from the left ventricle of the heart; the liver was excised, passed twice through 200ml washes of 0.17M-NaCl, wrapped in aluminium foil and immediately pressed between two blocks of solid CO_2. The elapsed time from the initial incision to the freezing of the liver was less than 60s. These manipulations did not significantly alter the specific radioactivity of the valine released from the tRNA (see Appendix).

Samples of blood were diluted with an equal volume of 0.2M-sodium citrate and spun immediately in a clinical centrifuge (1000*g*, 2min) to remove cells. An equal volume of ice-cold 20% (w/v) trichloroacetic acid was mixed with the chilled plasma. After 1h on ice, the supernatant was removed by centrifugation (730*g*, 10min) and frozen at −20°C.

Frozen livers were stored at −80°C until they were homogenized in 3 volumes of buffer (0.05M-cacodylate – 0.17M-NaCl – 0.33M-sucrose, pH6.0) with a Willems Polytron homogenizer. Samples were taken immediately into an equal volume of ice-cold 20% trichloroacetic acid and processed as described above for plasma. The remaining liver homogenate was used to isolate aminoacyl-tRNA by a modification of the procedure of Davey & Manchester (1969). This technique involved preliminary deproteinization with bentonite followed by cold phenol extraction and ethanol precipitation. Appropriate controls to establish the integrity and purity of this aminoacyl-tRNA preparation are presented in the Appendix.

The free valine concentration of plasma, liver homogenate and tRNA was determined with a Beckman 120C amino acid analyzer equipped with high sensitivity cuvettes as described by Airhart *et al.* (1973). Before application to the column, the plasma and liver samples were ether-extracted to remove the trichloroacetic acid and filtered through an Araflo ultrafiltration apparatus. The eluate from the column was automatically split into two fractions, one of which was mixed with ninhydrin for determination of the amino acids, the other was collected in scintillation vials in toluene containing 0.6% (w/v) 2,5-diphenyloxazole and 33% (v/v) Triton X-100. Radioactivity was monitored on a Beckman LS-100 liquid-scintillation counter. Quench correction was made by external standard; ^{3}H radioactivity was counted at about 15% efficiency in this system.

Because the liver receives 70% of its blood supply from the hepatic portal system and 30% from the arterial system (Elwyn *et al.*, 1968; Greenway & Stark, 1971), the specific radioactivity of the extracellular fluid actually bathing the liver cells was calculated as a composite of both portal and cardiac plasma in a ratio of 70/30. We felt justified in equating the extravascular and intravascular fluid because the hepatic sinusoids, each perfused by a mixture of portal and venous blood (Cohn & Pinkerton, 1969), have no permeability barriers to substances as large as plasma proteins and, within seconds, low-molecular-weight substances are equally distributed throughout the extracellular fluids (Goresky, 1970). The intimacy of each hepatocyte to the sinusoidal flow insures a rich and rapid perfusion and tends to eliminate the extracellular gradients characteristic of brain and muscle (Morgan *et al.*, 1961; Goresky, 1970; Yudilevich, 1970; Garlick, 1970). We have also assumed that the efflux of amino acids from the liver cells into the extracellular fluid does not significantly alter the measured specific radioactivity of extracellular valine. We have calculated that this dilution would decrease the extracellular specific radioactivity by no more than 12% even when we assumed the least favourable circumstances [instant equilibration between intracellular and extracellular fluids, maximal rate of intracellular degradation (Mortimore *et al.*, 1972) and minimal rate of plasma flow through the liver (Greenway & Stark, 1971)]. This in turn could

increase our estimate of the intracellular specific radioactivity by only about 4%. The extracellular fluid content of liver was measured 20min after intraperitoneal injection of $Na^{36}Cl$ (1μCi/0.3ml per 100g body wt.) (Schloerb & Grantham, 1965) and found to be equivalent to 0.27±0.01 (S.E.M., $n = 20$) ml of plasma per g of liver. The true specific radioactivity of the intracellular valine was then determined by subtracting the extracellular contribution of nmol and d.p.m. from the homogenate values.

[2,3-^{3}H]Valine (31.2mCi/μmol), [U-^{14}C]valine (265μCi/μmol) and $H^{36}Cl$ (40.8μCi/μmol) were obtained as sterile aqueous solutions from Amersham Searle, Chicago, Ill., U.S.A. $H^{36}Cl$ was neutralized with NaOH before injection. Bentonite was sized and deionized as detailed in the Appendix. Crystalline phenol was redistilled and saturated with buffer (0.05M-cacodylate–0.17M-NaCl, pH6.0) immediately before use.

Results and Discussion

After intraperitoneal administration of [^{3}H]valine to the intact rat, the specific radioactivity of portal plasma rose to a maximum within 5min (Fig. 1*b*). The specific radioactivity of cardiac plasma was 2–3 times lower and reached a peak between 5 and 10min. This difference resulted from unequal distribution of label, since the valine concentration was almost identical in cardiac and portal plasma (Table 1). As the [^{3}H]valine equilibrated with unlabelled free valine throughout the tissues, the specific radioactivity of cardiac plasma steadily approached that of portal plasma (Fig. 1*d*).

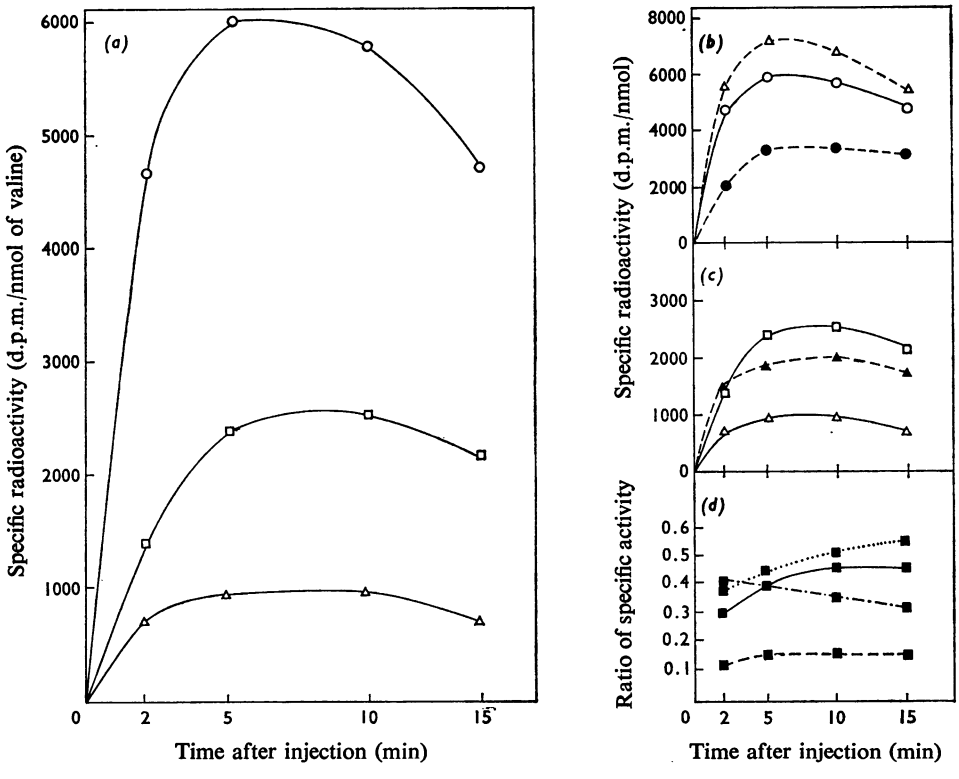

Fig. 1. *Appearance of [^{3}H]valine in various amino acid pools of the rat*

Administration of label and isolation and analysis of tissue samples are described in the text. (*a*) Relationship of the specific radioactivity of free valine from aminoacyl-tRNA (□) to those from the extracellular (○) and intracellular (△) pools. (*b*) Relationship of the calculated extracellular valine specific radioactivity (○) to the measured values of portal (△) and cardiac (●) plasma. (*c*) Relationship of the specific radioactivity of valine from aminoacyl-tRNA (□) to those of liver homogenate (▲) and intracellular (△) pools. (*d*) Ratios of the specific radioactivities of intracellular valine to extracellular valine (■---■); valine from aminoacyl-tRNA to extracellular valine (■——■); valine from cardiac plasma to portal plasma valine (■···■); and homogenate valine to extracellular valine (■-·-·-■).

Table 1. *Valine concentrations in various amino acid pools of the rat*

Measurements were made at 10a.m. on animals trained to feed from 6 to 10p.m. The fluid content of the samples was assumed to be 93% for plasma, 74% for homogenate and 47% for liver cells (Mortimore *et al.*, 1972). Correction for extracellular valine was made as described in the text. All values are expressed ±S.E.M. The number of determinations is given in parentheses.

	Concn. of valine (μM)
Portal plasma	190±6 (14)
Cardiac plasma	173±5 (14)
Extracellular fluid	185±3 (14)
Homogenate	256±11 (8)
Intracellular fluid	307±17 (8)
tRNA (nmol/g of liver)	0.63±0.04 (12)

The amino acids isolated in the liver homogenate originated from both extracellular and intracellular fluids within the liver. Correcting valine values in the homogenate for extracellular contamination gave an estimate of the intracellular specific radioactivity that was considerably lower than if the correction had not been made (Fig. 1*c*). For example, at 5min, the calculation involved subtracting 26% of the nmol of valine and 66% of the valine d.p.m. so that the resulting intracellular specific radioactivity was two-fold lower than that of the total homogenate. Nonetheless, the concentration of valine in the intracellular fluid was still greater than in the extracellular fluid (Table 1). Because the transport system for valine is non-concentrative (Oxender & Christensen, 1963), the increased intracellular valine suggests that a large proportion of intracellular amino acids was derived from protein degradation. This is consistent with other unpublished work from this laboratory (A. Vidrich, J. Airhart & E. A. Khairallah) indicating a general catabolism of liver protein at this time in the diurnal cycle of meal-fed rats. A substantial rate of protein degradation is also suggested by comparing the specific radioactivity ratio of the intracellular and extracellular fluids (Fig. 1*d*). The fact that the specific radioactivity of the intracellular valine does not exceed 16% of the extracellular value suggests that for every valine molecule entering the intracellular pool from the extracellular fluid, five others were contributed from degradation.

The uptake of labelled valine into aminoacyl-tRNA is compared with the uptake into both the intracellular and extracellular fluids in Fig. 1(*a*). As early as 2min the specific radioactivity of the valyl-tRNA was 2.5 times higher than that of the intracellular pool. Thus the total intracellular pool could not be the sole precursor of the valine charged to the tRNA. Further, the specific radioactivity of the tRNA was only half that of the extracellular fluid at equilibrium (Fig. 1*d*). If we assume that most of the aminoacyl-tRNA is metabolically active, this also suggests that the valine attached to the tRNA cannot be derived exclusively from the extracellular pool. Thus measurements of absolute rates of protein biosynthesis based on the specific radioactivity of either the extracellular or intracellular pool must be significantly in error.

This suggests that measurements of protein synthesis are accurate only when the specific radioactivity of the tRNA is determined directly. However, in certain unique circumstances this problem has been circumvented. Several investigators have expanded the extracellular pool to force amino acids into the intracellular pool and thus lessen the difference between the extracellular and intracellular specific radioactivities. Under these conditions either pool approximates to the specific radioactivity of the precursor (Mortimore *et al.*, 1972; Henshaw *et al.*, 1971; Morgan *et al.*, 1971). This technique has been useful, but there is always the danger that the artificially high concentrations of amino acids required to expand the pool might alter other physiological conditions. Further, since the specific radioactivity of the total homogenate may be close to that of the tRNA, other investigators have used the homogenate pool as the true precursor for protein synthesis (Loftfield & Harris, 1956; Morgan & Peters, 1971). In the present study we note that after 2min the specific radioactivity of the uncorrected homogenate was only about 20% below that of the valyl-tRNA (Fig. 1*c*). However, we must advise caution in applying this approximation too freely since we have found in experiments not presented here (A. Vidrich, J. Airhart & E. A. Khairallah, unpublished work) that the relationship varies with the hormonal and dietary state of the animal.

Since rat liver contains about 0.5mg of tRNA/g and since about 5% of the tRNA accepts valine (Allen *et al.*, 1969; Tidwell *et al.*, 1972), we would expect roughly 0.8nmol of valyl-tRNA/g of liver. Our yield is 0.63±0.04nmol/g. Since protein biosynthesis in rat liver has been estimated to be in the range of 30–120nmol of valine/min per g (Mortimore *et al.*, 1972; Morgan & Peters, 1971; Elwyn *et al.*, 1968; Swick, 1958; Stephen & Waterlow, 1966; Waterlow & Stephen, 1968; Gan & Jeffay, 1971), the half-life of valyl-tRNA in liver must be of the order of 0.3–2.0s. Thus it is highly improbable that a significant percentage of the aminoacyl-tRNA is metabolically inert. This rapid turnover is noteworthy since the specific radioactivity of valyl-tRNA did not reach maximum values relative to the extracellular fluid until about 10min even though the intracellular valine equilibrated completely with the extracellular pool within 5min (Fig. 1*d*).

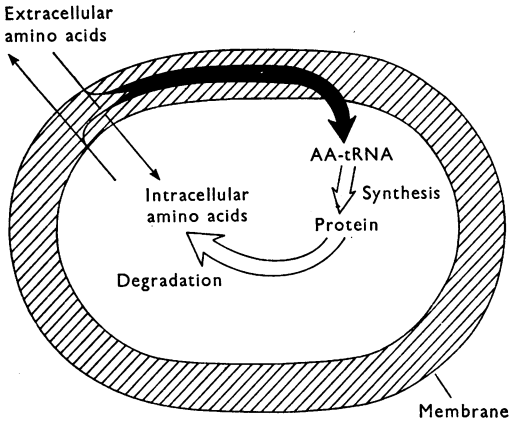

Fig. 2. *Model for compartmentation of amino acids for protein synthesis within the transport system of the cell membrane*

The observation that the specific radioactivity of the tRNA is intermediate between the liver extracellular and intracellular values suggests a mechanism for compartmentation involving the cell membrane system (Fig. 2). As amino acids from both the extracellular and intracellular fluids rapidly exchange across the cell membrane (Oxender & Christensen, 1963), they pass through a labile pool within the membrane (Hendler, 1962), and we propose that it is from this restricted site that the amino acids, used to charge the tRNA, originate. Since the transport system for valine is rapid but non-concentrative, the percentage composition of the membrane pool would be determined by the concentration gradient across the membrane. Thus the model would predict that the specific radioactivity of the tRNA would reflect the fact that the intracellular valine pool is 1.7 times as concentrated as the extracellular pool (Table 1). If indeed the acylated valine was derived from the membrane pool, then theoretically the tRNA should have a specific radioactivity of (extracellular specific radioactivity)(X)+(intracellular specific radioactivity)(1.7X) where X is equal to the percentage of the aminoacyl-tRNA contributed by the extracellular pool. At 15min this calculation predicts a specific radioactivity of the valyl-tRNA of 2218 d.p.m./nmol, and this is in excellent agreement with our experimental observation of 2170 d.p.m./nmol.

A detailed examination of Fig. 2 also leads to the prediction that there must be a functional interaction between the aminoacyl-tRNA synthetases and some component of the membrane system. Several laboratories have proposed that *in vivo* the synthetases as well as their corresponding tRNA species are organized in high-molecular-weight complexes with significant lipid content (Bandyopadhyay & Deutscher, 1971; Vennegoor *et al.*, 1972; Tscherne *et al.*, 1973). It has also been suggested that amino acids are transported across the membrane by phospholipid carriers (Hendler, 1958; Reiser & Christiansen, 1968), and as phospholipid exchange is rapid among membrane fractions (Zilversmit, 1971; Sauner & Levy, 1971), the lipid–amino acid complexes may facilitate equilibration between the plasma membrane pool and other internal membrane systems more proximal to the sites of protein synthesis. This conjecture may help explain the unexpected delay in equilibration of valine between the tRNA and the extracellular fluids noted above.

Our model predicts that amino acids within the membrane are used for protein synthesis regardless of whether they are entering or leaving the cell. This is different from the model proposed by Hider *et al.* (1969) who have suggested that only amino acids entering the cell from exogenous sources are taken up via a membrane pool before incorporation into proteins. However, attempts to apply these other models to predict rates of synthesis in rat liver have resulted in contradictions with the observed data (Mortimore *et al.*, 1972). The Hider *et al.* (1969) model has also been challenged by a recent report suggesting that changes in intracellular specific radioactivity strongly influence the specific radioactivity of precursor amino acids for protein synthesis in skeletal muscle (Li *et al.*, 1973). These authors have interpreted this as evidence that a homogeneous intracellular pool is the amino acid source for protein synthesis. However, without actual measurement of the specific radioactivity of aminoacyl-tRNA, their conclusions may not be fully justified.

There are several explanations for our data that do not presume the model in Fig. 2. It has been proposed that amino acids used in protein synthesis are simultaneously obtained from several different sources in the cell (Hendler, 1962). The tRNA itself may be compartmentalized so that some tRNA species charge directly from the extracellular milieu whereas others derive their amino acids from the general cell pool or some internal membrane pool. We find this rationale leads to models that are mathematically and experimentally cumbersome. However, we have not yet obtained data that confirm or eliminate this possibility. It has also been suggested by Mortimore *et al.* (1972) that there is a segregated amino acid pool within the liver that is derived from protein breakdown and supplies amino acids unidirectionally to the general cell pool and thus to protein synthesis. Their studies predict that under physiological conditions this segregated pool would amount to two-thirds of the total intracellular valine pool and is probably localized within the lysosomes (Woodside & Mortimore, 1972). This seems unlikely to us because there is evidence that not more than

7% of the total liver amino acids are located within the mitochondrial–lysosomal fraction (Portugal *et al.*, 1970). In our opinion the model of Fig. 2 not only represents a testable hypothesis, but also accommodates, as simply as possible, all the divergent data currently available on amino acid compartmentation.

Regardless of the theory eventually formulated to account for this phenomenon, the observation that a common intracellular amino acid pool is not the sole precursor of aminoacyl-tRNA reveals a significant gap in our knowledge of the topography of protein biosynthesis. This forces us to abandon the simplicity of the traditional view. However, the observation also points out certain structural and spatial relationships which we hope to use to understand eventually the orderly and efficient movement of raw materials within the cell and to evaluate the influence of nutritional and hormonal fluctuations on the procurement of amino acids for protein biosynthesis.

We acknowledge the excellent technical assistance of Mrs. Susan Sibiga and Mrs. Holly Sanders. We also thank Dr. H. H. Herrmann, Dr. N. Klein, Dr. A. Phillips, Dr. H. N. Munro and Dr. G. Wolf for helpful discussions of this problem. This study was supported by grants from the University of Connecticut Research Foundation and N.I.H. Grant AM 15919.

References

Airhart, J., Sibiga, S., Sanders, H. & Khairallah, E. A. (1973) *Anal. Biochem.* **53**, 132–140

Allen, R. E., Raines, P. L. & Regen, D. M. (1969) *Biochim. Biophys. Acta* **190**, 323–336

Alpers, D. H. & Thier, S. O. (1972) *Biochim. Biophys. Acta* **262**, 535–545

Bandyopadhyay, A. K. & Deutscher, M. P. (1971) *J. Mol. Biol.* **60**, 113–122

Cohn, J. N. & Pinkerton, A. L. (1969) *Amer. J. Physiol.* **216**, 285–289

Davey, P. J. & Manchester, K. L. (1969) *Biochim. Biophys. Acta* **182**, 85–97

Elwyn, D. H., Parikh, H. C. & Shoemaker, W. C. (1968) *Amer. J. Physiol.* **215**, 1260–1275

Fern, E. B. & Garlick, P. J. (1973) *Biochem. J.* **134**, 1127–1130

Gan, J. C. & Jeffay, H. (1971) *Biochim. Biophys. Acta* **242**, 125–135

Garlick, D. G. (1970) in *Capillary Permeability* (Crone, C. & Lassen, N. A., eds.), pp. 228–238, Academic Press, New York

Goldberg, A. L. (1971) *Proc. Nat. Acad. Sci. U.S.* **68**, 362–366

Goresky, C. A. (1970) in *Capillary Permeability* (Crone, C. & Lassen, N. A., eds.), pp. 413–430, Academic Press, New York

Greenway, C. V. & Stark, R. D. (1971) *Physiol. Rev.* **51**, 23–42

Haseltine, W. A. & Block, R. (1973) *Proc. Nat. Acad. Sci. U.S.* **70**, 1564–1568

Hendler, R. W. (1958) *Science* **128**, 143–144

Hendler, R. W. (1962) *Nature (London)* **193**, 821–823

Henshaw, E. C., Hirsch, C. A., Morton, B. E. & Hiatt, H. H. (1971) *J. Biol. Chem.* **246**, 436–446

Hershko, A., Mamont, P., Shields, R. & Tomkins, G. M. (1971) *Nature (London) New Biol.* **232**, 206–211

Hider, R. C., Fern, E. B. & London, D. R. (1969) *Biochem. J.* **114**, 171–178

Hider, R. C., Fern, E. B. & London, D. R. (1971) *Biochem. J.* **121**, 817–827

Jefferson, L. S., Koehler, J. O. & Morgan, H. E. (1972) *Proc. Nat. Acad. Sci. U.S.* **69**, 816–820

Kipnis, D. M., Reiss, E. & Helmreich, E. (1961) *Biochim. Biophys. Acta* **51**, 519–524

Li, J. B., Fulks, R. M. & Goldberg, A. L. (1973) *J. Biol. Chem.* **248**, 7272–7275

Loftfield, R. B. & Harris, A. (1956) *J. Biol. Chem.* **219**, 151–159

Manchester, K. L. (1970) in *Biochemical Actions of Hormones* (Litwack, G., ed.), vol. 1, pp. 277–283, Academic Press, New York

Manchester, K. L. & Wool, I. G. (1963) *Biochem. J.* **89**, 202–209

Martin, A. F., Prior, G. & Zak, R. (1973) *Fed. Proc. Fed. Amer. Soc. Exp. Biol.* **32**, Abstr. 532

Miller, L. L. (1962) in *Amino Acid Pools* (Holden, J. T., ed.), pp. 708–721, Elsevier Publishing Co., New York

Morgan, E. H. & Peters, T. (1971) *J. Biol. Chem.* **246**, 3500–3507

Morgan, H. E., Henderson, M. J., Regen, D. M. & Park, C. R. (1961) *J. Biol. Chem.* **236**, 253–261

Morgan, H. E., Earl, D., Broadus, A., Wolpert, E. B., Giger, K. E. & Jefferson, L. S. (1971) *J. Biol. Chem.* **246**, 2152–2162

Mortimore, G. E. & Mondon, C. E. (1970) *J. Biol. Chem.* **245**, 2375–2383

Mortimore, G. E., Woodside, K. H. & Henry, J. E. (1972) *J. Biol. Chem.* **247**, 2776–2784

Munro, H. N. (1970) in *Mammalian Protein Metabolism* (Munro, H. N., ed.), vol. 4, pp. 77–82, Academic Press, New York

Neidhardt, F. C. (1966) *Bacteriol. Rev.* **30**, 701–719

Oxender, D. L. & Christensen, H. N. (1963) *J. Biol. Chem.* **238**, 3686–3699

Portugal, F. H., Elwyn, D. H. & Jeffay, H. (1970) *Biochim. Biophys. Acta* **215**, 339–347

Reiser, S. & Christiansen, P. A. (1968) *J. Lipid Res.* **9**, 606–612

Rosenberg, L. E., Berman, M. & Segal, S. (1963) *Biochim. Biophys. Acta* **71**, 664–675

Sauner, M. & Levy, M. (1971) *J. Lipid Res.* **12**, 71–74

Schloerb, P. R. & Grantham, J. J. (1965) *J. Lab. Clin. Med.* **65**, 669–676

Smulson, M. E. & Thomas, J. (1969) *J. Biol. Chem.* **244**, 5309–5312

Stephen, J. M. L. & Waterlow, J. C. (1966) *Nature (London)* **111**, 978–980

Swick, R. W. (1958) *J. Biol. Chem.* **231**, 751–764

Tidwell, T., Bruce, B. J. & Griffin, A. C. (1972) *Cancer Res.* **32**, 1002–1008

Tscherne, J. S., Weinstein, I. B., Lanks, K. W., Gersten, N. B. & Cantor, C. R. (1973) *Biochemistry* **12**, 3859–3865
vanVenrooij, W. J., Poort, C., Kramer, M. F. & Jansen, M. T. (1972) *Eur. J. Biochem.* **30**, 427–433
vanVenrooij, W. J., Kuijper-Lenstra, A. H. & Kramer, M. F. (1973) *Biochim. Biophys. Acta* **312**, 392–398
Vennegoor, C., Stols, A. & Bloemendal, H. (1972) *J. Mol. Biol.* **65**, 375–378
Waterlow, J. C. & Stephen, J. M. L. (1968) *Clin. Sci.* **35**, 287–305
Woodside, K. H. & Mortimore, G. E. (1972) *J. Biol. Chem.* **247**, 6474–6481
Yudilevich, D. L. (1970) in *Capillary Permeability* (Crone, C. & Lassen, N. A., eds.), pp. 115–128, Academic Press, New York
Zilversmit, D. B. (1971) *J. Lipid Res.* **12**, 36–41

APPENDIX

Analysis of the Specific Radioactivity of Valine Isolated from Aminoacyl-Transfer Ribonucleic Acid of Rat Liver

By CAROL S. WALLYN, ALDA VIDRICH, JUDITH AIRHART and EDWARD A. KHAIRALLAH
Department of Biochemistry and Biophysics, University of Connecticut, Storrs, Conn. 06268, *U.S.A.*

Development of a technique for accurate measurement of the specific radioactivity of valine attached to the aminoacyl-tRNA *in vivo* was necessary to conduct the experiments presented in the main paper (Airhart *et al.*, 1974). This appendix gives the details of the isolation and analysis of our preparation as well as an assessment of the quality of the product.

Methods of isolation and analysis

Bentonite (Sigma Chemical Co., St. Louis, Mo., U.S.A.) was suspended in distilled water at 50g/l and magnetically stirred overnight. The coarsest particles were removed by centrifugation at 1450*g* for 15min. The cream-yellow uppermost layer of the first pellet was carefully scraped off and combined with the supernatant, and the rest of the pellet was discarded. The suspension was shaken manually and the 1450*g* centrifugation repeated. The uppermost layer of this second pellet was again retained with the supernatant and, after shaking, the suspension was centrifuged for 15min at 2500*g*; this was repeated once. After the second 2500*g* centrifugation, the pellet was completely discarded and the supernatant centrifuged at 9000*g* for 15min. The resulting pellet was washed twice with distilled water and resuspended in 0.1M-disodium ethylenediaminetetra-acetic acid and stirred overnight. Crude bentonite has a high metal ion content which decreases protein-binding capacity, and EDTA is effective in ion removal (Fraenkel-Conrat *et al.*, 1961; Watts & Mathias, 1967). The EDTA was removed by overnight dialysis against distilled water. The bentonite was recovered by centrifugation at 9000*g* for 15min and again washed twice with distilled water. The final pellet, which now had a grey–yellow colour, was resuspended in buffer A (0.05M-cacodylate–0.17M-NaCl, pH6.0) to a final bentonite concentration of 30–35mg/ml and stirred gently overnight. This bentonite suspension was stored at 4°C for up to a week or frozen at −17°C until needed.

Livers were obtained as described in the main paper and homogenized while still partially frozen in 3vol. of buffer B (0.05M-cacodylate–0.17M-NaCl–0.33M-sucrose, pH6.0) with a Willems Polytron PT-10 homogenizer for 1min at position 6. The following method for isolating aminoacyl-tRNA was adapted from the procedure of Davey & Manchester (1969) who discuss in detail the advantage of using cacodylate buffers and deproteinizing with bentonite. All steps were carried out at 4°C unless otherwise indicated. The homogenate was mixed with 0.15vol. of the bentonite suspension described above and centrifuged for 15min at 13000*g*. The resulting supernatant was decanted, again treated with bentonite and centrifuged as above. This procedure was repeated twice more or until the supernatant was clear and straw-yellow in colour. It was determined by the method of Lowry *et al.* (1951) that after the third bentonite treatment, the protein concentration of the supernatant was only about 1% of that of the original homogenate. The final supernatant was mixed with 0.075vol. of the bentonite suspension and centrifuged at 105000*g* for 1h. The resulting supernatant was mixed with an equal volume of cold redistilled phenol (saturated with 15% buffer A immediately before use), and this mixture was shaken at 4°C for 1h. After centrifugation at 25000*g* for 20min, the clear upper aqueous layer was removed with a chilled Pasteur pipette. A very small interphase was observed after the phenol treatment, obviating the need for several such extractions.